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(54) Title: TOTAL GONADOTROPAL ALPHA PEPTIDE CHAIN ASSAY

(57) Abstract

An assay procedure for predicting the onset of the ovulation in a human subject. Urine samples are obtained on consecutive days and assayed for total gonadotropal alpha peptide chain content. The alpha chain content surges to indicate ovulation and the onset of the fertile period.

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TOTAL GONADOTROPAL ALPHA PEPTIDE CHAIN ASSAY

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to assays for determining and/or detecting hormones in mammalian body fluids and in particular to assays for determining and/or detecting the luteinizing hormone surge which results in rupture of the preovulatory follicle and release of the ovum at ovulation. More particularly, the invention relates to the use of total gonadotropal alpha peptide chain content in urine as an enhanced indicator of luteinizing hormone content. Even more particularly, the invention relates to an immunoassay suitable for testing for constituents in human urine to determine the human fertile period, that is, the period in which viable sperm and a viable ovum may be present simultaneously in the female reproductive tract.

The Prior Art Background

For a number of reasons it may be clinically and/or diagnostically desirable to determine the presence and/or concentration of constituents in mammalian body fluids such as blood serum or urine. In some instances, mammalian hormonal activity and/or metabolism create situations where surges in concentrations of hormones or metabolites in body

fluids may b chronologically related to other events. In particular, a surge in the concentration of luteinizing hormones (LH) in human urine may be used to ascertain the fertile period of the menstrual cycle. For couples experiencing difficulty in conceiving there is a need for identifying the optimum period for intercourse or artificial insemination.

The human menstrual cycle is governed by the cyclical release of hormones from the female glands and organs. Such release is predictable and specifically related to ovulation by which ova are released from the ovaries and the lining of the uterus is made ready for pregnancy. Eventually, the released hormones and/or metabolites thereof find their way into the urine. The specific biological phenomena are described in detail in a published dissertation of Kevin J. Catt and John G. Pierce entitled "Gonadotropic Hormones of the Adenohypophysis", which appears as Chapter 3 (pp 75-114) of a treatise of Yen, S.S.C. and Jaffee, R.B., REPRODUCTIVE ENDOCRINOLOGY, 2d ed., W.B. Saunders, Philadelphia (1978). And suffice it to say, that during a normal menstrual cycle, the level of LH in female serum surges to cause the preovulatory follicle to rupture and release the ovum. This process is known as ovulation. The LH surge may be detected in female urine approximately 8 to 24 hours after the surge occurs in the blood. The surge of LH in human urine has thus been used as an indication that the fertile period is ongoing

or about to occur and a number of commercial assays for detecting the fertile period have been based on the measurement of LH concentrations in human urine.

Gonadotropins such as LH, follicle-stimulating hormone (FSH), human chorionic gonadotropin (hCG) and thyroid-stimulating hormone (TSH) are all well known hormones as discussed in the Catt et al. dissertation identified above. These hormones are all formed from two peptide chains (an alpha chain and a beta chain) which are non-covalently joined to present the intact hormone. The chains are capable of existing separately; however, they must be joined together to create a biologically active hormone. The alpha chains of LH, FSH, hCG and TSH are essentially identical but the beta chains are all different.

As is known in the art, the intact (holo) hormones and the free alpha and beta chains may be distinguished and assayed separately using various combinations of antibodies to private, public and/or conformational epitopes. In this regard, a public epitope is one that is accessible without regard to whether the chain is free or combined, a private epitope is one that is accessible only on free chains and a conformational epitope is one that is not available on either chain alone but only on an intact hormone. And, as indicated above, commercial fertile period assays have previously been configured to determine the LH surge by assaying for the intact hormone.

It has also been recognized that LH assays could potentially be directed to either total or free LH beta chain. However, there has been no suggestion in the prior art that there might be a sufficient correlation between preovulatory total alpha chain content and intact LH surges in urine to base a fertile period assay on total alpha chain. Even more so there has been no suggestion in the prior art that total alpha chain not only surges contemporaneously with LH but surges to a much greater level so that the assay is much more sensitive and precise.

SUMMARY OF THE INVENTION

In accordance with the invention it has been discovered that in the urine of normal cycling women the total content of gonadotropal alpha peptide chains in urine is considerably greater than the total LH beta chain or intact LH hormone content. It has also been discovered that the levels of alpha chain, beta chain and holohormone all increase in a coordinated fashion at mid cycle. Thus, it has been observed, in accordance with the invention, that the preovulatory surge of the holohormone is accompanied by a simultaneous magnified surge in total alpha chain. This facilitates construction of an assay having an enhanced signal to noise ratio compared to other assays based on the holohormone or on free or total beta chain. Measurements have shown that the molar equivalent concentration of gonadotropal alpha chain at the mid cycle

surge is approximately five to ten times greater than the molar equivalent concentrations of the holohormone or the beta chain.

To take advantage of the foregoing observed phenomena, the invention provides an assay procedure which includes the steps of obtaining a sample of body fluid from a mammal and analyzing said sample to determine or detect gonadotropal alpha peptide chains without regard to whether the latter are free or part of an intact hormone. In another aspect of the invention, an assay procedure is provided for determining preovulatory surging of luteinizing hormone which includes the steps of obtaining a plurality of samples of body fluid from a host and comparing the total gonadotropal alpha peptide chain content of said samples. In yet a further more specific aspect of the invention, an assay procedure is provided for predicting the onset of ovulation in a human subject which comprises obtaining a urine sample from a host on each of a plurality of consecutive time periods, analyzing each of said samples to determine total gonadotropal alpha peptide chain content thereof, and observing a surge in said total gonadotropal alpha peptide chain content as a prelude to ovulation.

In the more specific aspects of the invention an assay procedure is provided wherein the samples are analyzed using an immunological procedure. The immunological procedure may include the step of forming a sandwich using two different

antibodies, each of said antibodies being specifically bindable to a respective different binding site on the alpha chain.

In a preferred form of the invention, the assay procedure may include a sandwich ELISA procedure. And in a particularly preferred form of the invention, the samples are obtained on consecutive days and comprise first morning urine samples.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a chart which compares the mid cycle preovulatory surges of intact LH and total alpha chain on consecutive days of 7 cycles;

Figure 2 is a standard curve relating total alpha chain content to spectrophotometric absorbance values; and

Figure 3 illustrates the total alpha content of urine during essentially an entire menstrual cycle.

DETAILED DESCRIPTION OF THE INVENTION

As set forth above, the present invention is based on the discovery of the occurrence of two unexpected phenomena during the menstrual cycle of normally cycling women. First it was discovered that the traditional mid cycle surge of intact LH in the system that triggers ovulation is accompanied by a contemporaneous surge in the total level of gonadotropal alpha peptide chains in the system. Additionally it has been discovered that the alpha chain surge is greatly magnified relative to the surge of the holohormone.

These ph nomena were discovered by conducting dot blot analyses to determine the epitope specificity of certain LH antibodies. The antibodies investigated were HK1 2G9, LH25 2B10, INN-132, INN-72, LH26-2G9, WSP-2G5 and WSP-2B6. The INN antibodies are commercially available from I.D.C. (A-6080 Innsbruck/IGLS, Gsturnsteig 10, AUSTRIA) and the WSP antibodies are available commercially from Western States Plasma (3887 Alta Vista Drive, Fallbrook, CA). The other antibodies are available in-house at Hygeia Sciences, Inc.

LH antigens (free beta chain, free alpha chain and intact holohormone; obtained from Scripps) were bound to nitrocellulose at a concentration of 5 μg/ml in 0.2 M carbonate buffer (pH 8.0) via a 96-well device (Bio-Rad, Richmond, CA) by gravity filtration. The nitrocellulose was then exposed to a solution of 2% BSA in 0.2 M Carbonate, pH 9.0 to block remaining binding sites. The monoclonal LH antibodies at a concentration of 25 μg/ml, 100 μL/well were gravity filtered through a membrane. Unbound antibody was removed by washing the wells three times with 300 μL of 0.2 M Carbonate with 0.1% Tween 20 using vacuum filtration. Anti-mouse IgG labelled with Alkaline Phosphatase (Sigma Chemical, St. Louis, MO) diluted 1:1200 in blocking buffer, 100 μL/well, was gravity filtered, followed by three washes using the above mentioned wash buffer and vacuum filtration. A final wash with distilled water was used to remove any residual tween.

The membrane was removed from the device and exposed to substrate consisting of .01% Nitro Blue Tetrazolium, .004 M $CaCl_2$, and 0.5 mg/mL Indoxyl Phosphate in Veronal-Acetate Buffer, pH 9.6.

The results obtained were straight forward with very little background and it was determined that HK1 2G9 antibody is specific for a public epitope on the LH beta chain, LH25 2B10 antibody is specific for a public epitope on the LH beta chain, INN-132 antibody is specific for a public epitope on the alpha chain, INN-72 antibody is specific for a private epitope on the alpha chain, LH26-2G9 antibody is specific for a private epitope on the beta chain, WSP-2G5 antibody is specific for a public epitope on the alpha chain, and WSP-2B6 antibody is specific for a conformational epitope on the holohormone.

Pairs of these antibodies were thus used to develop sandwich ELISA assays for specific analytes. For example, WSP-2B6 (holohormone) and WSP-2G5 (alpha) could be used to assay for the intact hormone; INN-132 (alpha) and WSP-2G5 (alpha) could be used to assay for total alpha chain; and LH25 2B10 (beta) and HK1 2G9 (beta) could be used to assay for total beta chain.

Immunoassays were conducted using the foregoing pairs of antibodies and a 96 well Immulon plate. WSP-2B6, INN-132 and LH25 2B10 antibodies were used as the respective capture antibodies and the other antibody of each pair was labelled

with horse radish peroxidase (HRP). The capture antibodies were dispersed in 0.05 M PBS (pH 7.35) at different concentrations. The concentration of the WSP 2B6 antibody was 10 μg/ml; the concentration of the INN-132 antibody was 5 μg/ml; and the concentration of the LH25 2B10 antibody was 1 μg/ml. Each of these dispersions was coated (100 μl) in a plurality of wells and incubated overnight at room temperature.

For blocking, the plates were decanted and the wells were filled with 0.1 M Tris pH 8.0 containing 2% BSA and 20% Sucrose and incubated for at least 30 minutes. Prior to use the blocking solution was decanted and the plate was rinsed with tap water. For long term storage, the blocking solution could be decanted and the plate tapped several times onto paper towels to remove residual liquid and then placed in a vacuum desiccator.

The HRP labelled WSP-2G5 antibody was dispersed in 0.1 M Tris (pH 8.0) containing 1% bovine serum albumin (BSA), 1% polyethylene glycol (PEG-8000), and 0.1% Tween 20 at a concentration of 1 μg/ml, and the HRP labelled HK1 2G9 was dispersed at a concentration of 0.5 μg/ml. Urine samples were diluted 1:2 or greater in 0.1 M TRIS, 1% BSA.

The assays were conducted using 50 μl of diluted sample and 50 μl of HRP labelled antibody dispersion. Incubation was allowed to proceed for one hour at room temperature and then the plates were decanted and washed 6 to 8 times. Color was

developed using 100 µl of TMB substrate solution. The intensity of the color was detected on a spectrophotometric plate at absorbance 630 and results were evaluated using standard curves similar to the one presented in Fig. 2. The results for seven different panelists are set forth in Table I below and are summarized in Fig. 1. The data of Table I is set forth in terms of pica molar concentrations. From Table I and Fig. 1 it can be seen that total alpha chain content surges contemporaneously with the surge of intact LH and that the total alpha chain surge is much greater than the surges of other components.

TABLE I

SURGE COMPARISON

Panelist 0446

Cycle Day	LH Intact	Total Beta	Total Alpha	Free Alpha
12	0	0	106	0
13	0	0	114	0
14	75	121	1995	702
15	0	0	387	99
16	0	41	212	-

Panelist 0448

Cycle Day	LH Intact	Total Beta	Total Alpha	Free Alpha
9	0	0	88	-
10	0	37	242	46
11	138	112	1510	314
12	41	142	397	99
13	0	91	150	36

Panelist #449

Cycle Day	LH Intact	Total Beta	T tal Alpha	Free Alpha
10	0	0	176	-
14	0	0	140	-
15	0	0	254	-
16	0	45	350	107
17	55	49	398	127
18	184	169	1135	250

Panelist #450

Cycle Day	LH Intact	Total Beta	Total Alpha	Free Alpha
10	0	34	326	119
11	0	53	220	101
12	300	253	1970	667
13	146	161	1400	-
14	76	331	496	371

Panelist #451

Cycle Day	LH Intact	Total Beta	Total Alpha	Free Alpha
15	-	-	119	0
16	0	0	219	32
17	162	116	1055	198
18	0	49	203	42
19	0	236	290	57

Panelist #453

Cycle Day	LH Intact	Total Beta	Total Alpha	Free Alpha
10	0	0	179	-
11	0	0	312	64
13	68	95	2010	496
14	0	0	364	33
15	0	199	535	-

Panelist #454

Cycle Day	LH Intact	T tal Beta	T tal Alpha	Fr Alpha
14	0	55	347	-
15	0	0	149	54
16	207	172	1185	209
17	230	284	1062	159
18	88	376	468	111

The foregoing results are convincing and strongly prove that a fertile period assay may be based on an assay for total alpha chain. Such an assay is described in Example I hereinbelow.

EXAMPLE I

Total Alpha Chain Assay

Buffers

1. 0.1 M Tris, pH 8.0 with 1% Bovine Serum Albumin (BSA). This buffer is used to prepare alpha chain standards and as a diluent for the urine sample.
2. 0.1 M Tris, pH 8.0 with 1% BSA, 1% Polyethylene Glycol (PEG) of molecular weight 8000 and 0.1% Tween 20. This buffer is used as a diluent for the conjugates.

Antibodies

1. Anti-Alpha, designated INN-132. This antibody is used as the capture antibody.
2. Anti-Alpha, designated WSP-2G5. This antibody is against a different epitopic site than the first and will make a sandwich in the presence of alpha chain

consisting of the first antibody, the alpha chain and the second antibody. This system will measure both the free alpha chain and the alpha chain which is bound to beta chain. This second anti-alpha antibody is labelled with an enzyme, Horseradish Peroxidase (HRP) so that detection can be accomplished visually with the addition of a substrate or chromogen that will change color in the presence of HRP.

Plates

Immulon II 96 well polystyrene plates (Dynatech) are used for coating the capture antibody. The capture antibody is diluted in 0.05 M Phosphate Buffered Saline (PBS) to a concentration of 10 μg/mL. 100 μL is added per well and incubated overnight. The wells are decanted and then filled with a solution of 0.1 M Tris, pH 8.0 containing 2% BSA and 20% sucrose to block any remaining binding sites on the plastic wells. The blocking solution is incubated for a minimum of 30 minutes after which time the plate is decanted and rinsed with tap water. The plate is now ready for use.

Standards

For the total alpha assay, either purified alpha chain or purified intact LH can be used. Both are purchased from Scripps Laboratories, San Diego, CA. The concentrations used

are expressed in Molarity and range from 250 pM to 15.5 pM. Using these standards in an assay allows us to develop a standard curve from which unknown samples can be interpolated.

Tetramethylbenzidine (TMB) Substrate

TMB substrate is prepared using two solutions. The first, TMB solution, is prepared by adding 4.75 g of tetramethylbenzidine to 3.8 L of methanol. This solution should be protected from light. The second solution is substrate buffer with stannate and is prepared by admixing 5.35 g citric acid, 75.27 g sodium phosphate dibasic, 0.31 g sodium stannate, 5.2 mL 30% hydrogen peroxide and 0.26 g thimerosal in sufficient purified water to bring the total volume to 5.2 L. Final pH should be 4.9 to 5.1. This solution should never come in contact with metal. For use, 3 parts of the TMB solution is mixed with 7 parts of the substrate buffer.

Assay Format

50 μL of standard or diluted unknown urine is added in duplicate to wells. 50 μL of diluted anti-alpha conjugated to HRP is added to each test well. Incubation is for one hour at room temperature. The wells are then decanted and washed with tap water to remove any unbound labelled antibody. 100 μL of the chromogen substrate is then added. In the presence of HRP the clear substrate solution will change to a blue color. The amount of bound HRP labelled antibody is proportional to the concentration of alpha chain present in

either the standard or the unknown sample and therefore the color generated is in direct proportion to the alpha concentration. The intensity of the color solution is detected by a spectrophotometric plate reader at absorbance 630. A standard curve is drawn (Fig. 2) based on the average of the absorbance values (Table II) and the total alpha chain content of unknown samples is determined by interpolation from the standard curve and the pM multiplied by the dilution factor (Table III).

TABLE II

TOTAL ALPHA ASSAY

STANDARDS

[LH] pM	Absorbance 630	Average Absorbance Value
0.0	0.037 .015	.026
15.5	.071 .056	.064
31.0	.110 .108	.109
62.5	.206 .206	.206
125.0	.407 .448	.427
188.0	.694 .619	.656
250.0	.964 .952	.958

TABLE III

UNKNOWNS

Cycle Day	Dilution Factor	Absorbance 630	Average Absorbance Value	pM
2	2	.113 .107	.101	61
3	2	.431 .451	.472	264
4	2	.280 .240	.216	149
5	2	.201 .218	.209	127
6	2	.164 .188	.176	106
7	2	.153 .136	.144	85
8	2	.389 .340	.364	214
9	2	.147 .149	.148	88
10	2	.465 .362	.413	242
11	2	1.780 1.918	1.849	> linear
11	8	.669 .627	.648	1490
11	16	.315 .332	.323	1530
12	2	.666 .730	.698	397
13	2	.253 .275	.264	150
14	2	.273 .343	.308	183
15	2	.166 .170	.168	101
16	2	.179 .178	.178	108
17	2	.191 .218	.204	124
18	2	.107 .114	.110	63
19	2	.100 .100	.100	43

Cycle Day	Dilution Factor	Absorbance 630	Average Absorbance Value	pM
20	2	.184 .161	.172	104
21	2	.184 .162	.173	104
22	2	.117 .102	.109	62
23	2	.139 .144	.141	83
24	2	.342 .390	.366	215

The foregoing data is plotted in graphical form in Fig. 3 where it can be seen that the total alpha chain surge indicating the fertile period at day 11 is substantial.

WE CLAIM:

1. In an assay procedure, the steps of obtaining a sample of body fluid from a mammal and analyzing said sample to determine or detect gonadotropal alpha peptide chains without regard to whether the latter are free or part of an intact hormone.

2. In an assay procedure for determining preovulatory surging of luteinizing hormone, the steps of obtaining a plurality of samples of body fluid from a host and comparing the total gonadotropal alpha peptide chain content in said samples.

3. An assay procedure for predicting the onset of ovulation in a human subject comprising obtaining a urine sample from a host on each of a plurality of consecutive time periods, analyzing each of said samples to determine total gonadotropal alpha peptide chain content thereof and observing a surge in said total gonadotropal alpha peptide chain content as a prelude to ovulation.

4. An assay procedure as set forth in claim 3, wherein the samples are analyzed using an immunological procedure.

5. An assay procedure as set forth in claim 4, wherein said immunological procedure includes the step of forming a sandwich using two different antibodies, each of said antibodies being specifically bindable to a respective different binding site on said alpha chain.

6. An assay procedure as set forth in claim 5, wherein said immunological procedure comprises a sandwich ELISA procedure.

7. An assay procedure as set forth in claim 3, wherein said consecutive time periods are days and said urine samples are first morning urine samples.

8. An assay procedure as set forth in claim 6, wherein said consecutive time periods are days and said urine samples are first morning urine samples.

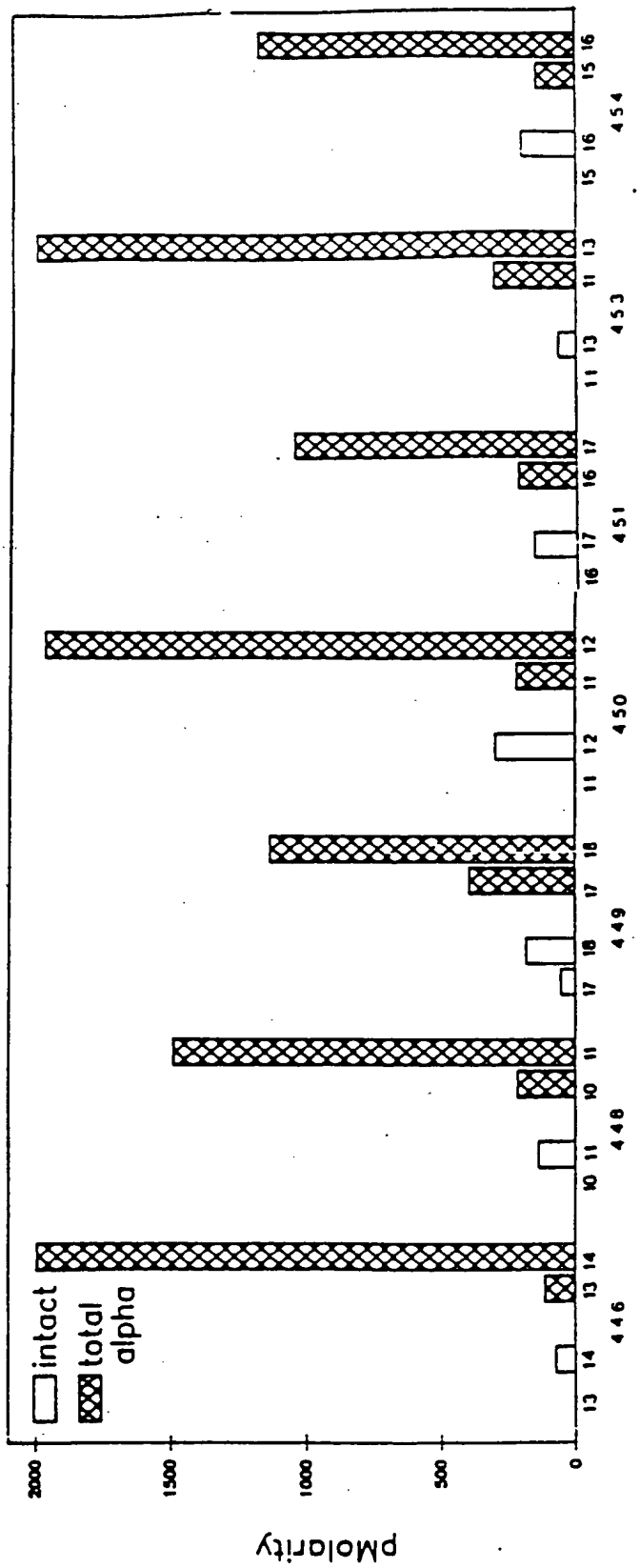
Surge Comparison Trials
intact
total alpha
pMolarity
2000
1500
1000
500
0
13 14 446
10 11 448
17 18 449
11 12 450
16 17 451
11 13 453
15 16 454
Two consecutive days of 7 cycles.

FIG. 1

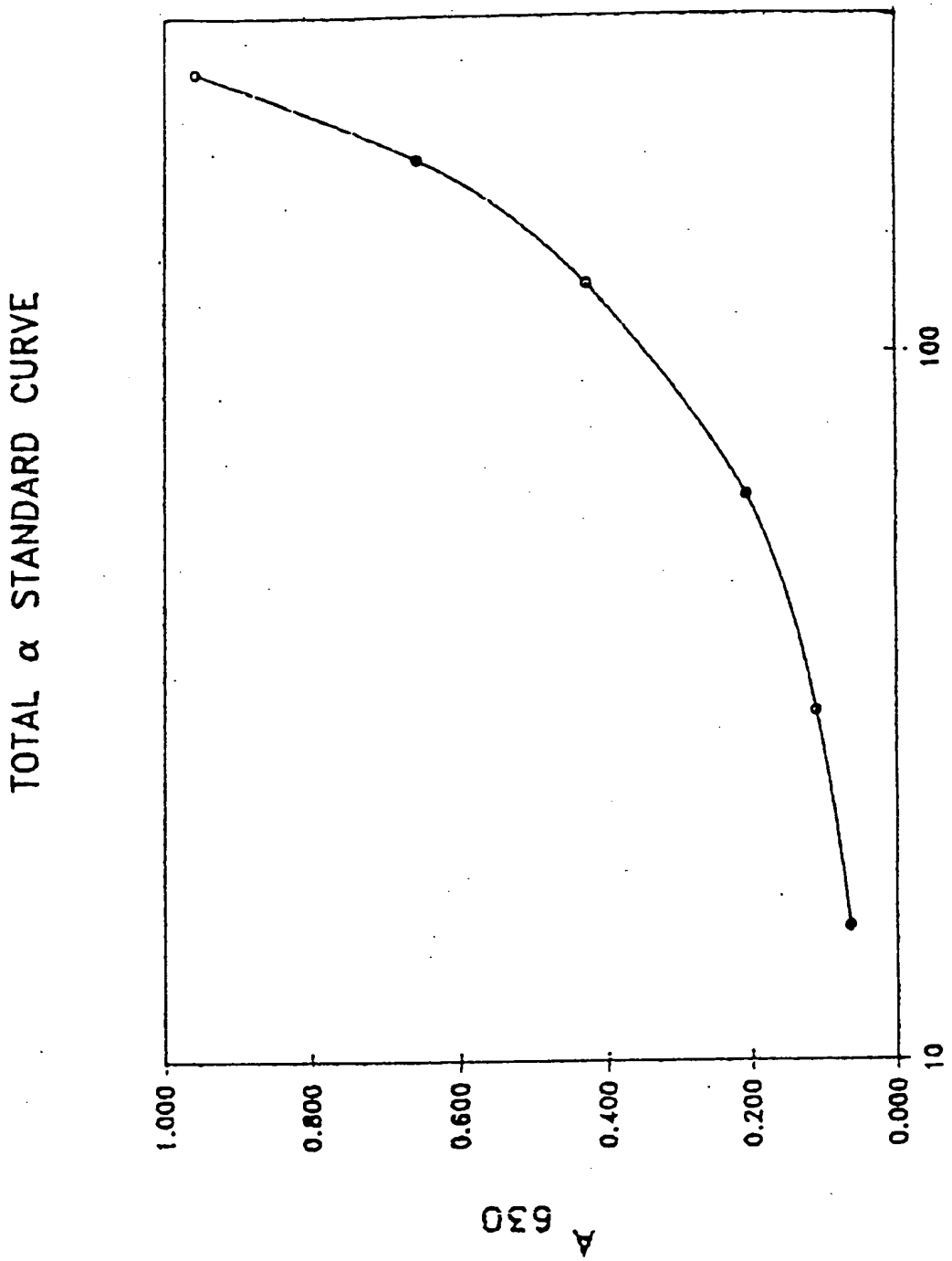
TOTAL α STANDARD CURVE
A 630
1.000
0.800
0.600
0.400
0.200
0.000
10
100
pM

FIG. 2

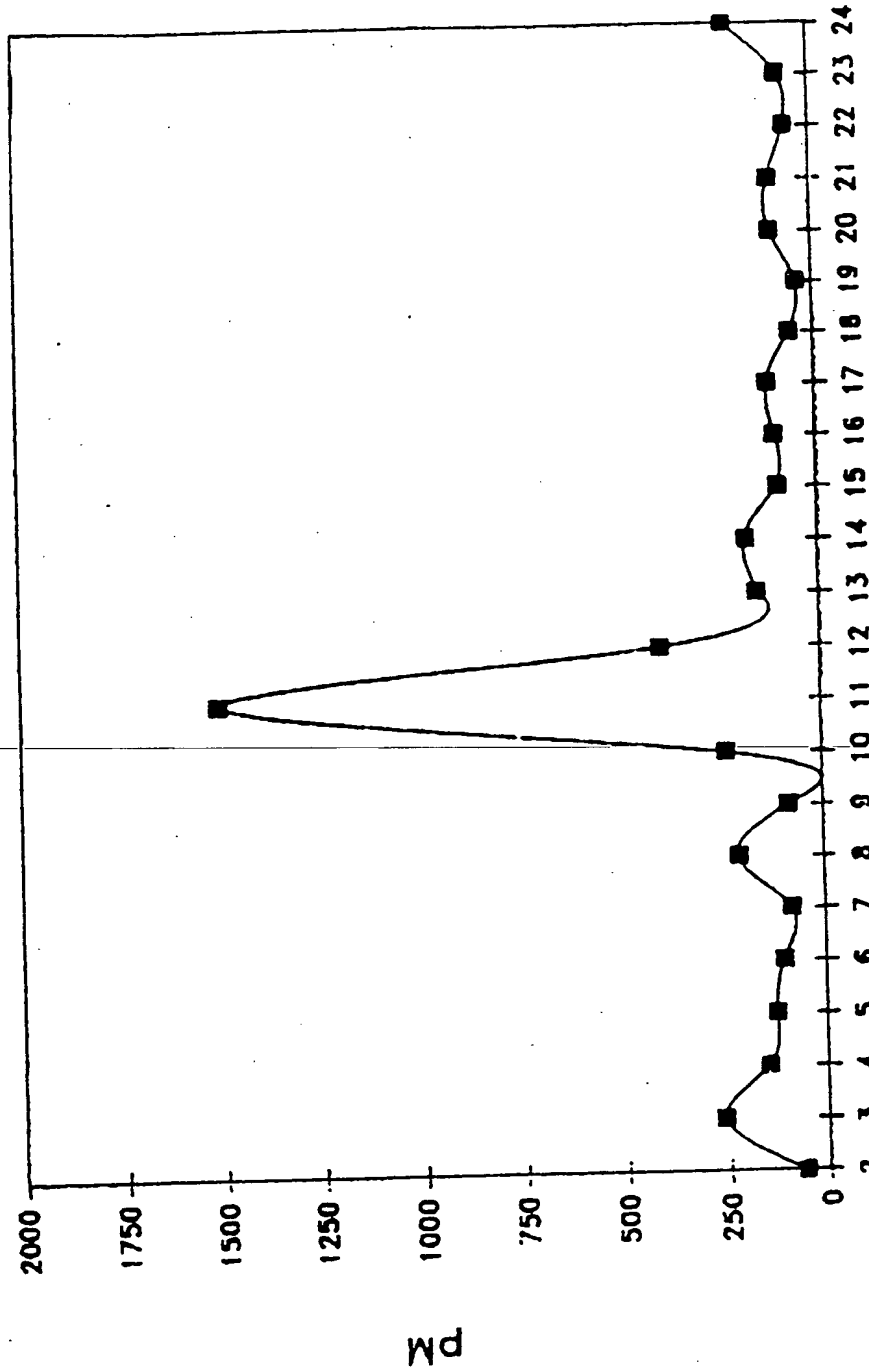
TOTAL α EIA
MONTH # 448
pM
2000
1750
1500
1250
1000
750
500
250
0
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
CYCLE DAY

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/02121

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C 12 Q 1/00
U.S. Cl: 435/7.1

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	435/ 4, 7.1, 7.92, 7.94, 28, 188, 805, 810

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	U.S.A. 4,659,678 (FORREST et al) 21 April 1987. See entire document.	1-8

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

26 April 1991

Date of Mailing of this International Search Report

18 JUN 1991

International Searching Authority

ISA/U.S.

Signature of Authorized Officer

Bradley L. Sisson

Bradley L. Sisson

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